Role of β_{IV}-spectrin in control of STAT3 signaling in cardiac fibroblasts

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Abstract

The fibroblast, a connective tissue cell, secretes or breaks down the extracellular matrix (ECM) via chemical signaling that alters gene expression. This function is necessary in cardiac fibroblasts to maintain the structure and function of the heart, but when an injury occurs and fibroblasts are active for remodeling, these changes may become maladaptive, leading to cardiac fibrosis and reduced cardiac function. Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that regulates genes involved in cell proliferation, survival, and synthesis of ECM components in fibroblasts. Preliminary studies from our group identified a novel complex involving the cytoskeletal protein β_{IV}-spectrin and STAT3, important for normal subcellular distribution and activity of STAT3 in the heart. Loss of β_{IV}-spectrin function promotes STAT3 mislocalization, changes in gene expression, and maladaptive cardiac remodeling with relevance to animal models of heart failure and human patients. While previous work has utilized a cardiomyocyte specific β_{IV}-spectrin knockout mouse to examine the role of spectrin/STAT3 complex in cardiomyocytes, the question remains whether this complex plays a broader role in non-myocytes (e.g. fibroblasts). Furthermore, questions remain about the mechanism by which STAT3 mislocalization results in altered gene transcription in any cell (myocyte or otherwise). The overall goal of this study is to better understand the relationship between changes in β_{IV}-spectrin function and altered gene transcription in the heart. This study tests the hypothesis that β_{IV}-spectrin associates with STAT3 in cardiac fibroblasts to directly regulate gene expression of proteins related to stress signaling and cardiac remodeling. The following mouse models were used for this study: wild type and *qv*^{4J} (lacks spectrin/STAT3 interaction). First, confocal microscopy was used to study the distribution of STAT3 in fibroblasts in the presence and absence of spectrin/STAT3 interaction. Next, echocardiography was used to assess cardiac function and heart sections were analyzed for fibrosis. Finally, quantitative PCR (qPCR) was used to quantify the expression of important STAT3 regulated genes in the fibroblasts. Results show STAT3 localization to the nucleus, decreased ejection fraction, increase in left ventricular (LV) chamber diameter, LV wall thinning, increased fibrosis, and increased mRNA expression of *COL14a* in the *qv*^{4J} mouse model compared to wild type. The mechanisms seen in cardiomyocytes and fibroblasts are potential targets for reducing or inhibiting remodeling effects.

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Table of Contents

List of Figures

Introduction

From 2009 to 2012, approximately 5.7 million Americans 20 years or older had heart failure (HF)[1]. The prevalence of HF is expected to increase by 46% from 2012 to 2030, which means more than 8 million people who are 18 years or older will have HF[1]. HF is a complex multifactorial syndrome characterized by maladaptive structural remodeling and compromised function of the heart. One cell type that plays an important role in the remodeling process is the fibroblast. Fibroblasts are flat, spindle-shaped, connective tissue cells that can be found in vertebrate organisms and are able to secrete or breakdown the extracellular matrix (ECM) depending on chemical signals, like cytokines and growth factors[2]. These signals can alter gene expression in fibroblasts and control the movement of cells to injured regions for healing and scar formation[2]. Fibroblasts convert to their activated form, myofibroblasts, to perform these functions[3]. For remodeling, myofibroblasts will secrete more ECM, which promotes cardiac fibrosis[3]. Cardiac fibrosis can lead to adverse effects, like cardiomyocyte hypertrophy and cell death[3].

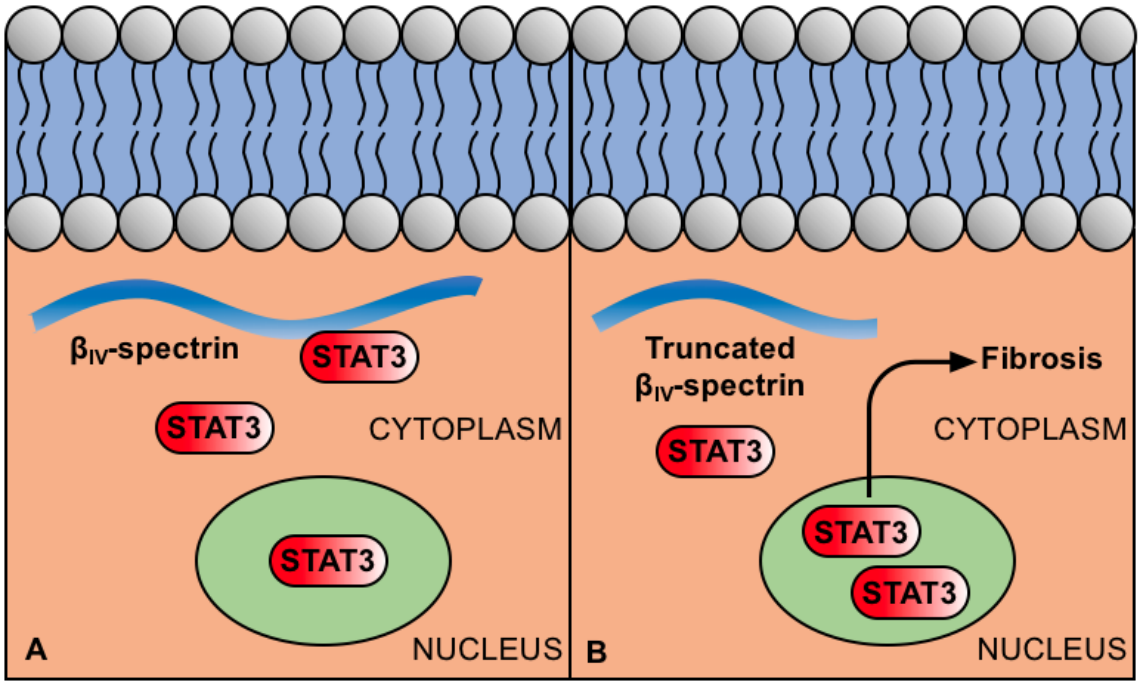


Figure 1. STAT3 associates with β_{IV}-spectrin in cardiomyocytes. (A) β_{IV}-spectrin targets STAT3 through direct protein-protein interaction and **(B)** the loss of β_{IV}-spectrin results in cellular redistribution of STAT3, changes in gene expression, increased fibrosis, and decreased heart function[5].

Signal transducer and activator of transcription 3 (STAT3) acts as a coordinator to balance protective and detrimental mechanisms of stress signaling in cardiomyocytes[4]. STAT3 is a transcription factor that regulates genes that code for proteins[4]. Specifically to fibroblasts, STAT3 contributes to cell proliferation, survival, and synthesis of ECM components[4]. Preliminary studies from our group have shown that STAT3 associates with β_{IV}-spectrin, a cytoskeletal protein, which controls the distribution and activity of STAT3 in cardiomyocytes[5]. β_{IV}-spectrin is primarily found at the cardiomyocyte intercalated disc, a specialized membrane domain important for cell-to-cell electrical and mechanical communication, and plays an important role in organizing local signaling domains for regulation of ion channels and cell membrane excitability in the heart[5,6,7]. Preliminary studies show that β_{IV}-spectrin targets STAT3 to the intercalated disc membrane through direct protein-protein interaction and that loss of β_{IV}-spectrin results in cellular redistribution of STAT3, changes in gene expression, increased fibrosis, and decreased heart function (Figure 1)[5]. Furthermore, downregulation of β_{IV}-spectrin and STAT3 redistribution was observed in mouse models of heart failure (transaortic constriction) and human heart failure patients[5]. Previous studies have shown that β_{IV}-spectrin

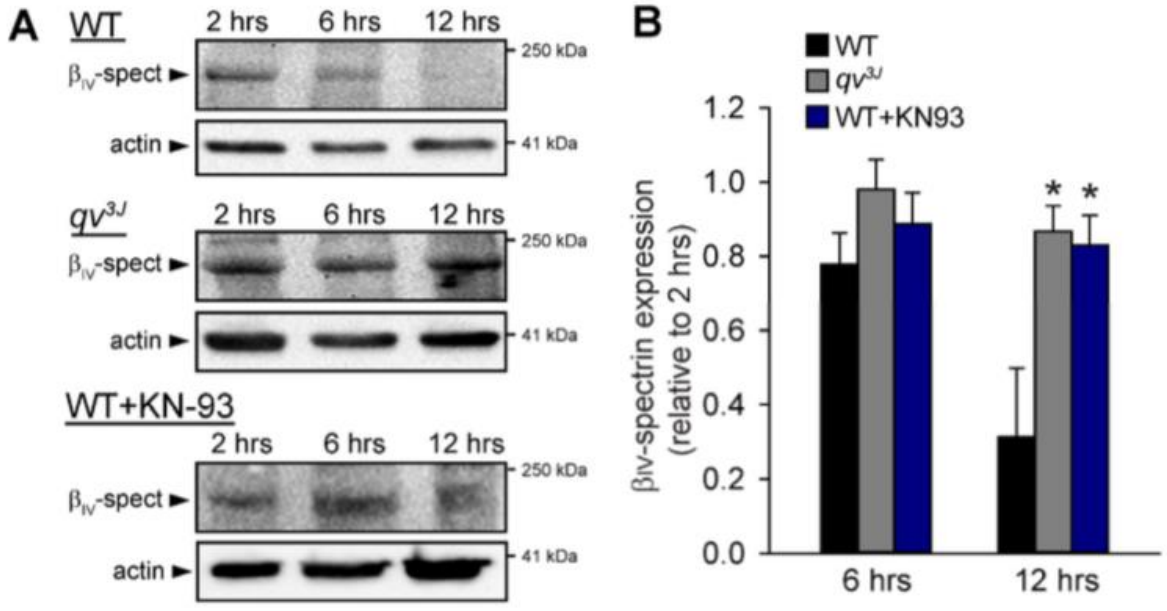


Figure 2. Long-term hyperphosphorylating conditions promote loss of β_{IV}-spectrin. Representative immunoblots **(A)** for β_{IV}-spectrin (actin used as loading control) for WT and *qv*^{3J} (lacks β_{IV}-spectrin/CaMKII interaction) subjected to prolonged pacing (2, 6, 12 hrs, 2 Hz) in the presence of isoproterenol and okadaic acid to simulate long-term (hyperphosphorylating) conditions. Subset of WT cells incubated with CaMKII inhibitor KN-93 (10 μm) during pacing. **(B)** Significant decrease in β_{IV}-spectrin expression at 6 and 12 hrs relative to baseline (2 hrs). *P<0.05 vs. WT, N=3 independent preparations per genotype/condition.

targets the multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) to ion channel substrates at the cardiomyocyte intercalated disc[6]. Hyperphosphorylating conditions to simulate long-term stress conditions promote CaMKII mediated degradation of β_{IV}-spectrin which may be prevented by global (CaMKII inhibitor, KN-93) or targeted (intercalated disc subpopulation, qv^{3J}) inhibition of CaMKII (Figure 2) [5].

Although it is known how STAT3 is distributed throughout cardiomyocytes via the spectrin/STAT3 association, and it is known that STAT3 alters gene expression in the nucleus, this study will directly link the spectrin/STAT3 association to gene expression. This study will test the hypothesis that β_{IV}-spectrin associates with STAT3 in cardiac fibroblasts to directly regulate gene expression of proteins related to stress signaling and cardiac remodeling by applying mechanisms seen in cardiomyocytes to fibroblasts, which are potential targets for reducing or inhibiting remodeling effects due to their pathology.

Methodology

Animals: *qv*^{4J} and wild type (WT) animals were obtained from Jackson Laboratories (Figure 3)[8]. All experiments were performed in 8-week-old male mice. Animals were euthanized using CO_2 and cervical dislocation followed by collection of tissue or cell isolation.

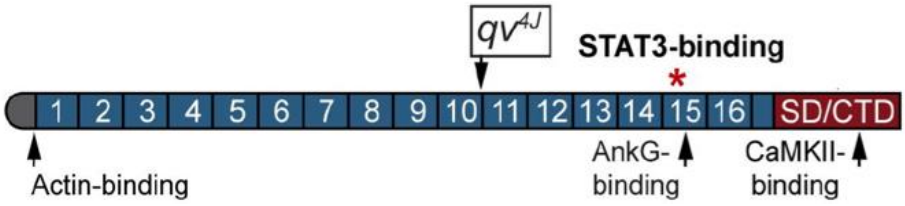


Figure 3. β_{IV}-spectrin structure in WT and *qv*^{4J} animals. *qv*^{4J} animals express mutant β_{IV}-spectrin allele with pre-mature stop codon prior to ankyrin-binding site. Spectrin repeat 15 contains a putative proline-rich STAT3-binding domain (*).

Isolation of Cardiac Fibroblasts: The following protocol was adapted from a previously used method[9]. Hearts were isolated from WT and *qv*^{4J} animals. The ventricles were excised and thoroughly minced with sterile fine scissors and digested in Ham's F-10 medium and Trypsin-EDTA (0.25%) at 37 °C for 30 min. During this incubation, the digesting tissue was triturated for a minute with a sterile serological pipette every 15 min. After incubation, a mixture of Ham's F-10 medium, collagenase type 2 (200 mg/ml), and soybean trypsin inhibitor (200 mg/ml) was added to the digesting tissue and incubated at 37 °C for 50 min. After digestion, the suspension of digested tissue was collected into a tube and debris was eliminated by two serial centrifugations at 2000 RPM for 5 min. Pellets were resuspended in media consisting of DMEM (1X), L-glutamine (200 mM), antibiotic-antimycotic (100X), and fetal bovine serum (10%). Cells were seeded onto a 12-well plate. Over the course of 4-6 days, media was replaced, and right before cells reached confluency, they were passaged into a 6-well plate, where they were allowed to reach confluency.

Confocal Microscopy*:* Cardiac fibroblast cells from WT and *qv*^{4J} were blocked in PBS containing Triton X-100 (0.15%), normal goat serum (3%), and BSA (1%). The cells were incubated in primary antibody overnight at 4 °C. Cells were then washed with PBS and then incubated in secondary antibody for 2 hrs at room temperature. The cells were mounted using Vectashield with DAPI and coverslips. Confocal microscopy images were collected on a Zeiss

780 confocal microscope [Objective W Plan Apochromat 40 x/1.0 DID (Zeiss), pinhole equals 1.0 Airy Disc] with Carl Zeiss Imaging software.

Echocardiography: Echocardiography images of the left ventricle were collected on WT and *qv*^{4J} mice using the Vevo 2100 (VisualSonics) with the MS-400 transducer. Animals were anesthetized and secured in the supine position. The heart was first imaged in the long-axis view, which was used to determine the position of the heart and serve as a reference point to obtain the short-axis view. Short-axis M-mode was used to assess cardiac function. M-mode images were used to calculate heart rate (HR), LV inner chamber diameter in diastole (LVID, d), ejection fraction (EF), fractional shortening (FS), LV anterior wall thickness in diastole (LVAW, d), and LV posterior wall thickness in diastole (LVPW, d).

Fibrosis: Left ventricular tissue from WT and *qv*^{4J} animals were obtained and stained using Masson's Trichrome method. Custom software was used to calculate the percentage of fibrosis of interstitial and epicardial regions.

Quantitative PCR: The following protocol was adapted from a previously used method[7]. Total RNA from WT and *qv*^{4J} cardiac fibroblasts was extracted with TRIzol Reagent plus RNeasy column purification (Qiagen) following manufacturer's instructions. 500 ng of total RNA treated with DNase I was used for the first-strand complementary DNA synthesis using SuperScript III reverse transcriptase VILO cDNA Synthesis Kit (Invitrogen). qPCR reactions were performed in triplicates on cDNA samples in 96-well optical plates with SYBR® Green Assay (Thermo Fisher) and SYBR® Green Master Mix (Thermo Fisher) to maximize PCR precision and uniformity. PCR was performed at 95 °C for 3 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min on Applied Biosystems 7900HT Fast Real-Time PCR System or StepOnePlus Real-Time PCR System (Life Technologies). PCR data were analyzed using the relative standard curve method and the 2 delta Ct method was used to calculate fold changes in relative gene expression. PCR products were confirmed by the melt-curve analysis, amplicon length, and DNA sequencing. *RPL-7* levels were used as a normalization control.

Statistics: Sigmaplot 13.0 was used for statistical analyses. *P*-values were determined for

comparison using Student's t-test. A *P*-value of <0.05 was considered statistically significant.

Results

STAT3 localization is altered in qv^{4J} cardiac fibroblasts lacking spectrin/STAT3 interaction

Preliminary studies have shown that the loss of β_{IV}-spectrin results in altered subcellular localization of STAT3 in cardiac myocytes[5]. In order to test whether β_{IV}-spectrin serves a similar function in cardiac fibroblasts, the heart was isolated from a *qv^{4J}* animal and processed to grow fibroblasts. Confocal images show increased localization of STAT3 to the nucleus in the *qv^{4J}* fibroblast compared to that of the WT cardiac fibroblast (Figure 4). These results indicate that the loss of β_{IV}-spectrin in cardiac fibroblasts alters the distribution of STAT3 due to the loss of spectrin/STAT3 interaction in the *qv^{4J}* cardiac fibroblast.

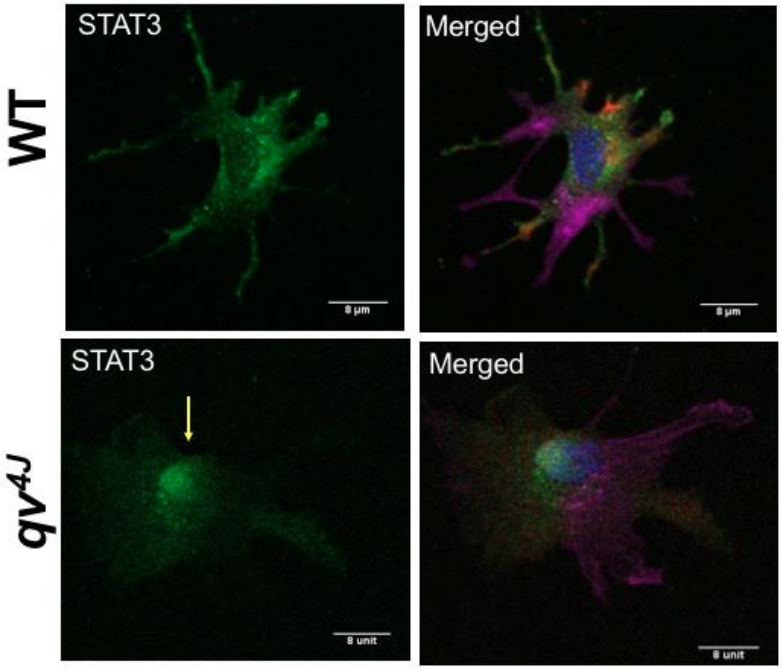
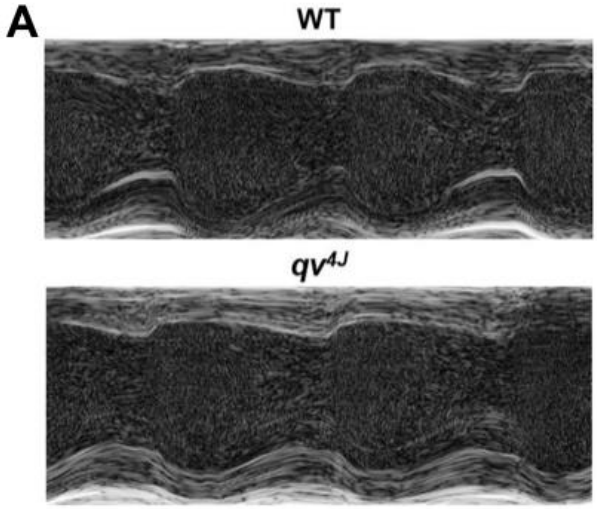


Figure 4. Altered STAT3 localization in *qv^{4J}* cardiac fibroblasts lacking spectrin/STAT3 interaction. Representative confocal microscopy images of permeabilized adult WT and *qv^{4J}* CFs immunostained for STAT3 (*green*), phalloidin (*purple*), and DAPI (*blue*). Scale bar = 8µm.

Cardiac function is compromised in qv^{4J} mice lacking spectrin/STAT3 interaction

Preliminary studies have shown that the loss of β_{IV}-spectrin results in decreased heart function[5]. In order to verify this mechanism specifically in *qv^{4J}* animals, echocardiography was used to assess cardiac function. *qv^{4J}* animals demonstrated symptoms of heart failure, including LV dilation and decreased ejection fraction as well as LV wall thinning compared to WT at baseline

conditions (Figure 5). Consistent with previous findings, these results show that the lack of spectrin/STAT3 interaction in the *qv*^{4J} animal contributes to decreased cardiac function.



B

	WT (N=5)	qv4J (N=5)
HR (bpm)	446.71±26.44	392.144±51.95
LVID,d (mm)	2.98±0.07	3.34±0.19
EF (%)	63.24±1.33	49.49±1.91*
FS (%)	33.244±0.82	24.336±1.05*
LVAW,d (mm)	0.798±0.04	0.619±0.04*
LVPW,d (mm)	0.78±0.17	0.632±0.06

HR = heart rate;
LVID,d = left ventricular inner chamber diameter in diastole;
EF = ejection fraction;
FS = fractional shortening;
LVAW,d = LV anterior wall thickness in diastole;
LVPW,d = LV posterior wall thickness in diastole
*P<0.05 vs. WT

Figure 5. Compromised cardiac function in *qv*^{4J} mice lacking spectrin/STAT3 interaction. Representative echocardiograms **(A)** and summary data **(B)** from 8-week old WT and *qv*^{4J} mouse at baseline. *qv*^{4J} mice show decreased ejection fraction, increase in left ventricular (LV) chamber diameter, and LV wall thinning compared to WT at baseline conditions. *P<0.05 in *qv*^{4J} vs. WT, N=5.

Fibrosis increases in qv^{4J} *mice lacking spectrin/STAT3 interaction*

Preliminary studies have shown that the loss of β_{IV}-spectrin results in increased fibrosis[5]. Analysis of interstitial and epicardial regions of heart sections using a custom software was used

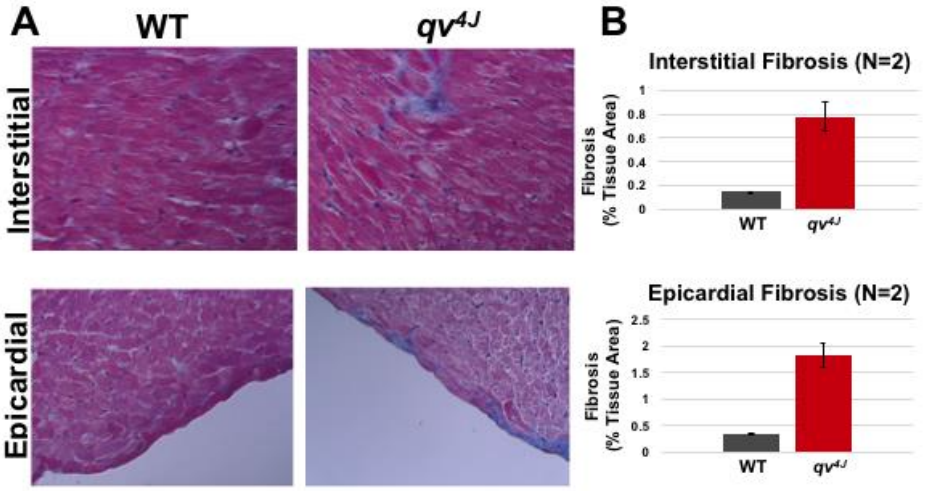


Figure 6. Increased fibrosis in *qv*^{4J} mice lacking spectrin/STAT3 interaction. (A) Representative Masson's Trichrome stained heart sections (collagen labeled blue). **(B)** Summary data showing fibrosis as percentage of tissue area from WT and *qv*^{4J} at baseline conditions. N=2 independent preparations. 18 fields analyzed per preparation.

to verify this mechanism specifically in qv^{4J} animals. Results showed increased fibrosis in qv^{4J} hearts, both in the interstitial and epicardial regions, compared to that of WT hearts (Figure 6). These results suggest that the lack of spectrin/STAT3 interaction in the qv^{4J} animal contributes to maladaptive cardiac remodeling, which may then be leading to decreased heart function.

Gene expression is altered in qv^{4J} cardiac fibroblasts lacking spectrin/STAT3 interaction

To determine whether β_{IV}-spectrin dysfunction was associated with altered gene expression in cardiac fibroblasts, quantitative PCR (qPCR) was performed on WT and qv^{4J} cardiac fibroblast populations. qPCR confirmed the expression of *SPTBN4* in WT cardiac fibroblasts and the lack of expression of *SPTBN4* in qv^{4J} cardiac fibroblasts. mRNA expression of *COL14a* in qv^{4J} cardiac fibroblasts was found to be greater than that of WT cardiomyocytes. This is consistent with the idea that fibroblasts play a direct role in cardiac remodeling through the secretion of the ECM in response to chemical signaling. qv^{4J} cardiac fibroblasts show higher expression of *COL14a* and lower expression of *SPTBN4* compared to those of WT cardiac fibroblasts. These results support that gene expression is altered in qv^{4J} cardiac fibroblasts because they lack spectrin/STAT3 interaction.

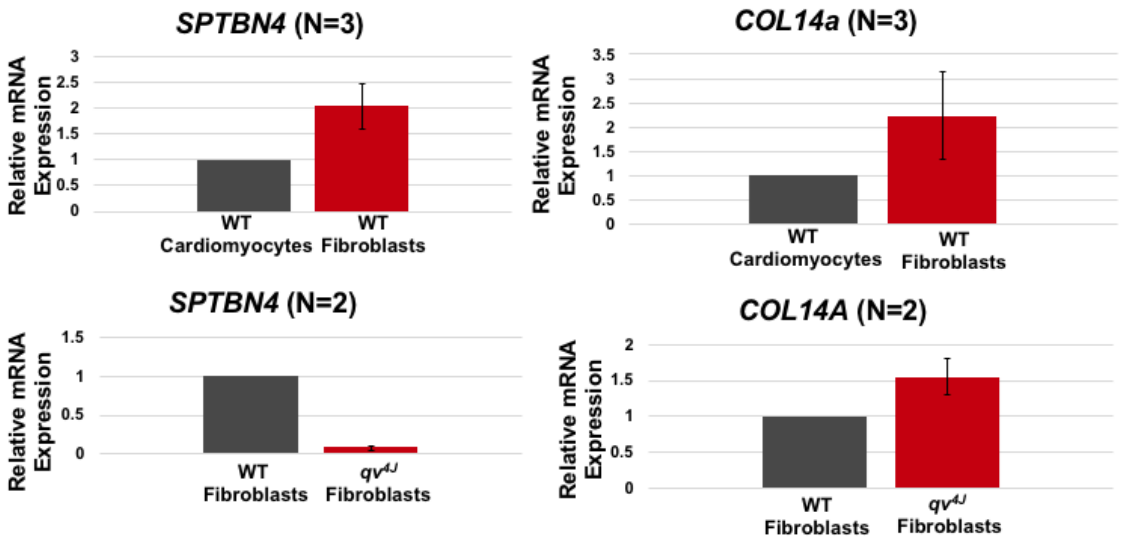


Figure 7. Altered gene expression in qv^{4J} cardiac fibroblasts. qPCR shows *SPTBN4* mRNA expression in WT CFs with increased *COL14a* mRNA in CFs compared to cardiomyocytes. qv^{4J} CFs show higher *COL14a* and lower *SPTBN4* mRNA expression compared to WT CFs. *RPL-7* was used a control gene.

Conclusion

Preliminary studies from our group identified a complex involving the cytoskeletal protein β_{IV}-spectrin and STAT3, important for normal subcellular distribution and activity of STAT3 in the heart[5]. Loss of β_{IV}-spectrin function promotes STAT3 mislocalization, changes in gene expression, and maladaptive cardiac remodeling with relevance to animal models of heart failure and human patients[5]. The overall goal of this study was to better understand the relationship between changes in β_{IV}-spectrin function and altered gene transcription in the heart. This study tested the hypothesis that β_{IV}-spectrin associates with STAT3 in cardiac fibroblasts to directly regulate gene expression of proteins related to stress signaling and cardiac remodeling. The results from this study show that spectrin/STAT3 complex is present in cardiac fibroblasts, and the loss of spectrin/STAT3 interaction leads to the localization of STAT3 to the nucleus (Figure 4). Results suggest that the spectrin/STAT3 complex may play a role in regulating cardiac fibrosis (Figures 5 and 6). It was also shown that β_{IV}-spectrin associates with STAT3 in cardiac fibroblasts to regulate gene expression of proteins related to stress signaling and cardiac remodeling (Figure 7). These mechanisms seen in cardiomyocytes and cardiac fibroblasts are potential targets for reducing or inhibiting remodeling effects. Inhibiting the activation of STAT3 in cardiomyocytes and/or cardiac fibroblasts may decrease cardiac fibrosis, thereby reducing adverse effects such as hypertrophy and cell death that contribute to heart failure.

For future work, it will be important to study the functional activity of STAT3 in fibroblasts as well as quantify the presence of STAT3 in cytoplasmic and nucleic fractions of fibroblasts. It will also be important to show that the changes in STAT3 distribution and activity in cardiac fibroblasts are due to its direct interaction with β_{IV}-spectrin through rescue experiments. The potential for using spectrin/STAT3 as a therapeutic target can be studied through treatments with STAT3 inhibitor. Finally, obtaining a mouse model with a cardiac fibroblast-specific β_{IV}-spectrin knockout will be important for distinguishing the effects of the spectrin/STAT3 interactions in cardiac fibroblasts from those of cardiomyocytes.

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